

Laboratory investigation of *Pneumocystis carinii* pneumonia

J M W Chatterton, D O Ho Yen

Introduction

Unexpected *Pneumocystis carinii* pneumonia (PCP) led to the identification of the acquired immunodeficiency syndrome (AIDS). The laboratory diagnosis of *P carinii* infection has had a less auspicious start. As this fungus is not easily grown, there has been a tendency for physicians to make a clinical diagnosis. Early and rapid confirmation of the diagnosis of PCP is important and the development of sensitive and specific tests has made such evaluation possible.¹ This paper will critically examine the different types of specimen and the laboratory techniques used for the identification of *P carinii*. The role of serology will be discussed.

The fact that the vast majority of AIDS patients develop PCP during their illness² has been a great stimulus to research into PCP. Yet, it must be remembered that PCP is also a major problem for other immunocompromised patients in whom the pathology may be different, and other laboratory tests such as serology may be more relevant.^{3,4} Consequently diagnosis of *P carinii* infection in AIDS and non-AIDS patients will be considered. In the future newer laboratory techniques may increase the sensitivity of diagnosis. Recently the polymerase chain reaction has been applied to the diagnosis of PCP with considerable success.⁵ However, all of the laboratory techniques, both new and old, have advantages and disadvantages. In addition, the use of prophylaxis and greater awareness of PCP by clinicians will result in earlier diagnosis, and the role of laboratory tests in this changing environment needs careful assessment. This paper attempts to identify a rational approach to the investigation of *P carinii* infection in terms of the types of specimens and tests.

Specimens

There are essentially three types of specimen for the demonstration of *P carinii*: sputum, bronchoalveolar secretions and biopsy material. Each type of specimen has had a wide range of diagnostic success in different

studies⁶⁻¹⁹ (table 1). In addition, serum specimens may be helpful and are separately considered.

1 Sputum

The diagnostic success of spontaneously expectorated sputum has been poor.²⁰ One review of cases of PCP in patients with immunocompromise, malignancy and transplants estimated the yield of correct diagnosis to be 6%.²¹ Although some believe sputum examination is not worthwhile,²² in AIDS patients where there is a much higher infestation²³ this may still be useful. *P carinii* has been identified in 8/33 (24%) routine sputa from episodes of PCP in AIDS patients,⁶ with a diagnostic sensitivity of 57%.

As the cough in PCP is usually dry and non-productive, sputum production can be induced by inhalation of hypertonic saline produced by an ultrasonic nebuliser.¹ Examination of induced sputum has been positive in between 55%¹⁷ and 92%⁸ of AIDS patients with confirmed PCP. Sputum induction is non-invasive but requires careful supervision and rigorous patient preparation.²⁴ The procedure is not without difficulty. In a recent prospective study of 151 AIDS patients 28 were unable to produce a specimen, 29 provided an inadequate sample, 17 found the procedure unpleasant, 15 had nausea and vomiting, eight became dyspnoeic, three had intractable cough and one acute bronchoconstriction.²⁴ More serious complications have been reported in particular high risk patients. Rapid and fatal pleural effusions developed after sputum induction in four AIDS patients with Kaposi's sarcoma and small pleural effusions.²⁵ It has been shown that the use of experienced dedicated personnel increases the number of successful attempts at sputum induction but does not increase the diagnostic yield.²⁴

2 Bronchoalveolar lavage (BAL)

Bronchoscopy with lavage is readily available in most hospitals and carries a low morbidity. After inspection of the airways the bronchoscope is wedged into a subsegmental bronchus, usually in the right middle lobe, and 100 mls of sterile saline is instilled in 20 ml aliquots. Aspiration after each aliquot should yield 40-50 mls of lavage fluid. The commonest complication in a study of 171 AIDS patients was fever, and BAL was well tolerated in the 23 patients considered to be at high risk of complications.¹⁸ When 72 AIDS patients who underwent sputum induction and BAL were questioned, 64 preferred BAL.²⁴ The procedure can be successfully used in paediatric

Department of Microbiology,
Raigmore Hospital,
Inverness IV2 3UJ, UK
J M W Chatterton
D O Ho Yen

Correspondence to
Dr Ho Yen

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Table 1 Relative success of sampling procedures for demonstration of *P carinii*⁶⁻¹⁹

Procedures	Procedures positive/total	Overall success (%)	Range (%)
Spontaneously expectorated sputum	11/81	14	6-24
Induced sputum	275/536	51	15-92
Bronchoalveolar lavage	300/524	57	42-89
Bronchoalveolar lavage	*78/398	20	0-21
Bronchial washings	159/260	61	60-79
Transbronchial biopsy	245/341	72	57-93

All AIDS patients except *which includes samples from 49 immunocompromised and 18 AIDS patients.

patients²⁶ despite significant tachypnoea and or hypoxia.¹⁵ Variable success has been reported in AIDS patients (table 1) and this may reflect differences in sampling techniques or experience in demonstrating *P. carinii*. Yields have been lowest in studies which have included non-AIDS patients (table 1). This is not surprising since PCP is much more common in AIDS than in other immunosuppressed patients and the lower cyst burden in non-AIDS patients makes demonstration of *P. carinii* more difficult. To be successful lavage needs to be properly carried out ensuring adequate alveolar sampling. When properly performed BAL should sample the alveolar contents of an entire subsegment of lung.²²

3 Biopsy

Transbronchial biopsy (TBB) may be performed after BAL if that procedure does not provide a diagnosis.² The complications of fiberoptic bronchoscopy however, are markedly increased when TBB is performed.²⁷ The procedure carries a risk of haemorrhage and pneumothorax. Broaddus *et al.*¹⁸ found pneumothorax occurred in 23/253 (9%) transbronchial biopsies in AIDS patients and in 15 tube thoracostomy was required to expand a collapsed lung. Miller *et al.*²⁷ reported a 16% pneumothorax rate in 74 HIV antibody positive patients after TBB and one death from massive haemorrhage. The technique would not be recommended in thrombocytopenic patients or those requiring ventilation.¹⁸ Transbronchial biopsy has generally been more successful than BAL for diagnosing PCP but similar success rates have also been reported (table 1). One study,²⁸ found TBB was considerably less successful than BAL, with sensitivities of 56% and 90% respectively. However, this analysis included 16/60 (27%) TBB specimens which were considered "unsatisfactory" because of the small quantity of alveolar tissue. In one case PCP was only diagnosed by TBB. Transbronchial biopsy provides material for diagnosing other causes of respiratory distress such as tumours,²⁰ and this advantage may justify the higher associated morbidity in some cases.

Biopsy can also be performed transthoracically by percutaneous needle aspiration. Diagnostic yields of more than 80% have been reported, but may be associated with significant morbidity. In a series of 27 cancer patients there was a 30% incidence of complications: eight pneumothorax, one massive haemorrhage and two deaths directly related to the

procedure.²⁹ In AIDS patients sudden death because of coexisting autonomic neuropathy has been reported.³⁰ While needle biopsy is blind, open lung biopsy offers the advantage of exploration and direct vision. It is the "gold standard" sampling procedure.^{1, 22} A generous tissue specimen can be taken and it is unlikely that the laboratory will find the specimen inadequate or unsatisfactory.²⁹ However, it requires general anaesthetic, and has major complications of pneumothorax, pneumomediastinum and bleeding. Consequently it is reserved for those patients who deteriorate clinically despite conventional therapy or in whom bronchoscopy with lavage fails to secure the diagnosis.

Demonstration of *P. carinii*

A variety of chemical and immunological stains have been used to identify *P. carinii*; however, there can be major difficulties in the interpretation of the results of these stains.

1 Chemical staining

These fall into two groups: cyst wall stains and trophozoite/sporozoite stains. The most popular cyst wall stain is the Gomori (Grocott) methenamine silver (GMS) but toluidine blue O, cresyl echt violet and Gram-Weigert have all been widely used. Under ideal conditions all provide high contrast and easy recognition of distinctive *P. carinii* cysts thus affording rapid analysis.³¹ The literature is full of technical modifications to reduce time and improve results. These stains can be used on all clinical samples including tissue sections.²² Use of a single cyst wall stain however will result in false negatives (table 2). When GMS and Gram-Weigert were used to examine 65 BAL specimens 48 positives were detected. If GMS alone had been used 4/48 (8%) would not have been detected.⁷ There has recently been considerable interest in the use of Calcofluor white for demonstrating *P. carinii*. This chemofluorescent agent binds to polysaccharides in fungi and other organisms emitting bright apple green fluorescence. It is claimed to be a rapid, sensitive and accurate method of demonstrating *P. carinii* cysts in BAL,³² but its use in sputum remains to be evaluated.

Sporozoite/trophozoite stains such as Giemsa, polychrome methylene blue, Wright, Gram and Diff-Quik are rapid and easy to perform. Unfortunately they are not selective. Since parasite and tissue elements are equally stained, the morphologically unremarkable sporozoites/trophozoites are difficult to identify. Although trophozoites may outnumber cysts by 100:1 in clinical specimens, screening is time consuming and requires considerable experience.³¹ These stains are suitable for respiratory fluids and imprints, but not for tissue sections.³¹ Demonstration of foamy alveolar casts with Papanicolaou stain has proved helpful in detecting *P. carinii* in BAL.¹⁹ Papanicolaou stains were positive on 7/65 (11%) BAL specimens that were negative by GMS, and where *P. carinii* was later confirmed by other methods. Methods that stain both

Table 2 Comparison of different staining techniques for demonstration of *P. carinii*^{6, 8, 11-14}

Staining Method	Procedure	Sensitivity* (%)	Average (%)
Diff-Quik	IS	76,92	83
	BAL	81	81
Toluidine blue O	IS	80,92	83
	BAL	100,100	100
Gomori (Grocott)	IS	74,92	83
methenamine silver	BAL	67,86,89	82
Monoclonal	IS	92,97,100,100	97
immunofluorescence	BAL	86,93,96,100,100	94

IS = induced sputum; BAL = bronchoalveolar lavage

* (total positive by one stain/total positive by all stains used) × 100.

cysts and trophozoites/sporozites have been published.³³ Pleasing results can be achieved but can be difficult to interpret.²² It has recently been recommended that at least three individual stains (GMS, toluidine blue O and Giemsa) should be used to examine clinical specimens.²⁶

2 Immunological staining

In the 1980s reports of the successful use of monoclonal antibodies for demonstrating *P. carinii* appeared.^{6,8,13} Several antibody clones are now available which are specific for *P. carinii*. Some recognise cysts (R13/3G4-6, 5E12); others both cysts and trophozoites (2E3, 3F6 2G2).³⁴ Used in immunofluorescence tests (IFA), singly and in cocktails these antibodies have proved to be powerful tools giving the highest success in clinical specimens (table 2). Elvin *et al.*⁶ demonstrated *P. carinii* in 14/43 sputum specimens by IFA when only 3/14 were demonstrated by GMS. Other studies have confirmed this superiority.^{8,14} When 182 specimens (124 induced sputum, 56 BAL, one transbronchial biopsy and one lung tissue) were examined by GMS, toluidine blue O, Diff-Quik and IFA, Ng *et al.*⁶ found 17 which were only positive by IFA. Diagnostic kits incorporating different monoclonal antibodies are now commercially available. Commercial kits are expensive and the technique occasionally fails (table 2). Nevertheless monoclonal antibodies offer the advantages of improved sensitivity, high specificity and less time is required for specimen examination.

3 Interpretation

While too stringent morphological criteria may produce up to 49% false negatives,⁹ there are other problems in interpretation. Other pathogens may be confused with *P. carinii*, especially the yeast-like fungi *Histoplasma capsulatum* var *capsulatum*, *Cryptococcus neoformans* and *Torulopsis glabrata*.²² The specificity of *P. carinii* monoclonals virtually eliminates this problem, but we and others¹¹ have found that the IFA may occasionally stain fungal elements and budding yeast forms. Ideally 3–5 or more well-defined cysts should be seen.^{9,14} Fluorescent material resembling trophozoites must not be interpreted as positive⁹ but should provoke an intensive search for cysts.¹² More than one smear should be examined. Gill *et al.*¹² found four specimens initially negative by toluidine blue O and five negative by IFA were positive on second smear evaluation. Inclusion of *P. carinii* controls is advisable, as inconsistent uptake of GMS may give false negatives when fungal controls are positive.³⁵ We routinely include a *P. carinii* seeded sample of any specimen we examine. On several occasions we have failed to demonstrate *P. carinii* in the seeded specimen by IFA when the kit control provided was satisfactory. Awareness of the problems is the key to success. Use of a combination of staining methods and experience will minimise problems of interpretation and maximise demonstration of *P. carinii*. It should always be remembered that *P. carinii* can persist in the lungs of patients even after

treatment for PCP.² Consequently in subsequent episodes of pneumonia due to other pathogens *P. carinii* may still be found in BAL.

Serology

Failure to develop reliable in vitro culture systems for *P. carinii* has seriously hindered the development of serological tests. A wide variety of antigens prepared from infected lung have been used in immunofluorescent antibody and ELISA systems. Much of the published evidence has concluded antibody testing is not worthwhile for sporadic disease in the immunocompromised.^{20,22} While controversial, the value of serological testing should not be dismissed too readily. In 20 patients with PCP, including patients with transplants, malignancy and AIDS, we found 65% (13/20) were positive for antibody compared with 9% (13/148) of Scottish blood donors.⁴ The present tests are far from satisfactory, yet they may still have a role to play and we continue to offer antibody testing. Testing is relatively simple and demonstration of seroconversion or rising titres can provide a presumptive diagnosis.³

Purification and exploitation of specific antigens will hopefully allow the development of more effective serological tests. A 95 kDa protein identified as a major surface antigen of human *P. carinii* isolates has been purified and used in an IgG ELISA system.³⁶ In 38 controls with no history of PCP (27 infants, eight healthy laboratory workers and three immunocompromised patients) two were possibly antibody positive. Among eight AIDS and non-AIDS immunocompromised patients with no history of PCP, only one had antibody; among 30 with a history of PCP, 10 (33%) were antibody positive and seven possibly positive. When serial samples were examined some patients developed gp95 antibody after clinical disease, while others developed no detectable antibody despite multiple episodes of PCP. In a subgroup of patients gp95 antibody was demonstrated before PCP was diagnosed. The authors have suggested this may be of predictive value in PCP. Other workers³⁷ have used immunoblotting to monitor the serological responses among AIDS patients with one or more episodes of PCP. In 41% (7/17) of patients with single episodes of PCP and 93% (13/14) of patients with recurrent PCP, active IgM and/or IgG antibody responses to a 40 kDa antigen were detected. Demonstration of this serological response has rekindled interest in serology as an epidemiological tool.

Identification of *P. carinii* specific antigen in serum would allow current infection to be diagnosed. Initially promising results were obtained using polyclonal antisera in ELISA, counterimmunoelectrophoresis and latex tests but these proved insensitive.³⁸ Reliable differentiation of clinical PCP from subclinical infection is not yet possible. However antigenaemia has been found in patients with PCP before the appearance of antibody and the onset of acute pneumonia.³⁹

Investigation of PCP

The best approach to the investigation of PCP depends on many factors. The degree of immunocompromise, the severity of illness in the patient, the expertise in obtaining non-invasive specimens, the availability of invasive procedures, and the expertise of the laboratory are critical considerations. However, one of the most significant factors in reducing the mortality of PCP in the last 5 years has been increased medical and patient awareness of the condition,⁴⁰ leading to earlier diagnosis and treatment. A scheme for the investigation of patients with suspected PCP is depicted in the figure.

Although the clinical presentation of PCP is not diagnostic, the condition should be suspected in individuals with immunocompromise who are short of breath, have a fever and a dry cough (fig). Part of the difficulty in diagnosis is the spectrum of clinical findings: fever is present in about 90% of cases, breathlessness in 80% and a dry cough in 50%.²⁰ Among HIV infected individuals, illness may be insidious and respiratory failure may occur in patients presenting later or among children. Increasingly, PCP will be found in HIV infected individuals, and the infection should be considered in patients at high risk of HIV. When T helper (CD4) cells are below $200 \times 10^6/l$, there is severe immunocompromise and a high chance of developing PCP.⁴¹

The vast majority of patients with respira-

tory complaints will have a chest radiograph. Classically in PCP, there will be diffuse, bilateral changes.² In 5% of patients changes are unilateral²⁰ and atypical radiographic appearances including cystic changes and cavitating and non-cavitating pulmonary nodules have been recorded.²⁻²⁰ In 5–10% of AIDS patients there can be a normal chest radiograph.² Therefore, in patients with continuing symptoms further investigation is indicated (fig). Measurement of arterial blood gases is useful in assessing the severity of pulmonary involvement.² Hypoxia is frequent but not inevitable in PCP.²⁻²⁰ Abnormalities, irrespective of radiological findings, should institute further investigations in any high risk patient (fig). Additional, non-specific evidence of lung disease may be obtained by lung function tests or gallium lung scans.² The single-breath diffusing capacity for carbon monoxide appears to be the most sensitive for PCP.

After establishing that the patient has lung disease, it seems logical to first use non-invasive techniques to establish the diagnosis. In exceptional cases, because of the patient's history, clinical features and severity of illness, it may be more appropriate to proceed to invasive techniques. The diagnostic success of sputum is poor but in patients who spontaneously expectorate sputum, the sample is easily obtained and can be examined for other causes of the patient's symptoms (fig). However, many PCP patients have a non-productive cough and for these patients induced sputum may be the investigation of choice. The problem with induced sputum is its variable success (table 1). Success probably depends on the expertise of the staff and the use of the correct type of nebuliser. If the procedure is rarely performed, it is best to proceed to bronchoalveolar lavage, especially as patients can find this less traumatic than sputum induction.²⁴

Unfortunately, many laboratories do not provide a serological service for the diagnosis of PCP. Serological investigations are rapid and have the advantage of an easily obtainable specimen. Seroconversions or rising titres allow the diagnosis to be made³ and toxic treatment started. However, serology is probably best used in patients who are being closely monitored.

The high sensitivity and specificity of bronchoalveolar lavage, with its low level of complications probably makes this the invasive method of choice. This procedure may also help in the diagnosis of pulmonary haemorrhage, other infection, tumour infiltrate or indicate drug-induced pneumonitis.²⁰ Transbronchial biopsy may be complicated by haemorrhage or pneumothorax and is particularly indicated if malignancy is suspected. Lung biopsy is reserved for very special situations,²⁻²⁰ such as clinical deterioration, negative investigations (fig), or uncorrectable coagulopathy.

The future

As has been demonstrated in this paper, the type of specimen influences diagnostic success. The trend away from invasive procedures has

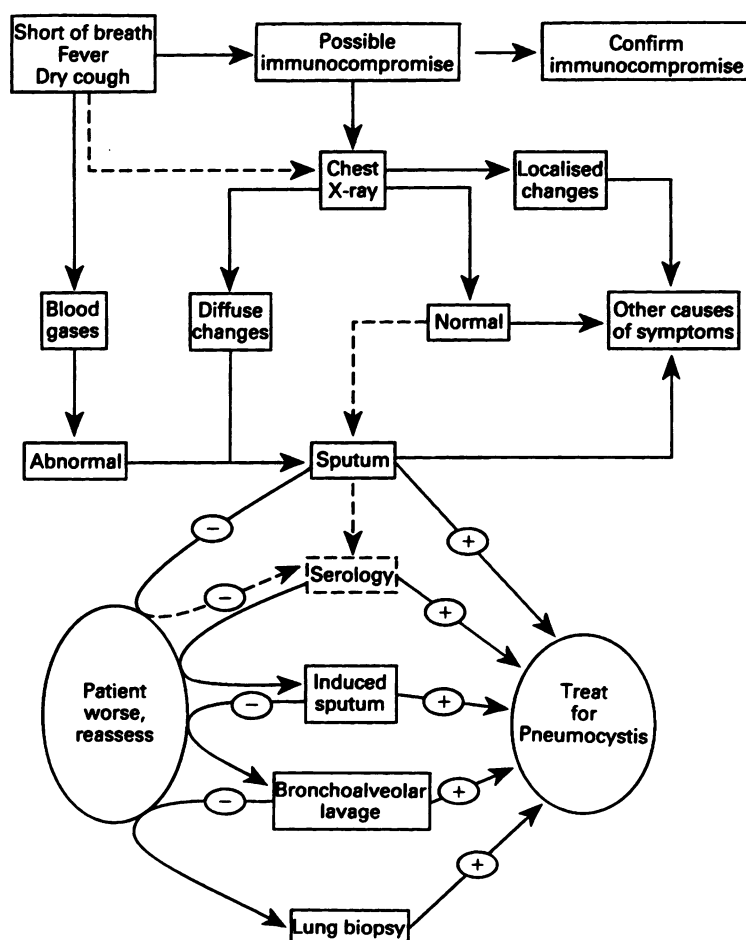


Figure Investigation of *P. carinii* pneumonia

been justified by the high concentration of *P. carinii* in PCP in AIDS patients and the consequent ease of detection in non-invasive samples.²² However, the medical management of immunocompromised individuals is changing with an emphasis on prevention. Criteria for primary and secondary prophylaxis for PCP have been established.⁴⁰ In addition, in HIV infected individuals early treatment with zidovudine may prevent the development of the severe immunocompromise of AIDS.⁴² As it becomes necessary to diagnose PCP earlier when there is a lower concentration of *P. carinii*, laboratory tests will have to be more sensitive.

In the last decade, the progression from chemical stains to monoclonal antibodies has increased the specificity and sensitivity of laboratory diagnosis. Further development of specific staining procedures, especially incorporating anti-trophozoite as well as anti-cyst elements will improve diagnostic success. However, it is with the use of molecular biology based tests that sensitivity will be best increased. DNA probes have already been successfully used in hybridisation studies⁴³ and DNA amplification by polymerase chain reaction has proved to be a highly sensitive and specific technique for detecting *P. carinii* in BAL⁴⁴ and induced sputum.⁴⁵ With the scenario of greater patient and medical awareness, prophylactic treatment, better drugs, and more sensitive detection methods invasive diagnostic procedures will only be used in a minority of patients.

There has been concern that much of the research into *P. carinii* infection has concentrated on the cyst rather than the trophozoite stage of the life cycle.³⁸ It was suggested that trophozoites are more important as they outnumber cysts in alveolar lavage, drugs effective in PCP have a greater effect on trophozoites, and the number of cysts does not correlate with the severity of PCP. More work is required to identify antigenic differences between cysts and trophozoites.

To date, much has been learnt about the diagnosis of PCP. Sampling techniques have improved, laboratory tests have been refined and a scheme for the investigation of PCP can be suggested. Although in the future there will be further progress, a logical approach to the investigation of PCP is likely to be the most important factor in reducing morbidity and mortality from this infection.

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- Kovacs JA. Advances in the diagnosis of *Pneumocystis carinii* pneumonia. *AIDS Clin Rev* 1990;129-47.
- Hopewell PC. *Pneumocystis carinii* pneumonia: diagnosis. *J Infect Dis* 1988;157:1115-9.
- Chatterton JMW, Joss AWL, Williams H, Ho-Yen DO. *Pneumocystis carinii* antibody testing. *J Clin Pathol* 1989;42:865-8.
- Ho-Yen DO, Chatterton JMW, Joss AWL. *Pneumocystis carinii* pneumonia. *BMJ* 1988;297:855.
- Kitada K, Oka S, Kimura S, et al. Detection of *Pneumocystis carinii* sequences by polymerase chain reaction: animal models and clinical application to noninvasive specimens. *J Clin Microbiol* 1991;29:1985-90.
- Elvin KM, Bjorkman A, Linder E, Heurlin N, Hjerpe A. *Pneumocystis carinii* pneumonia: detection of parasites in sputum and bronchoalveolar lavage fluid by monoclonal antibodies. *BMJ* 1988;297:381-4.
- Chechani V, Allam AA, Haseeb MA, Kamholz SL. *Pneumocystis carinii* pneumonia in patients with AIDS: evaluation of lavage and staining techniques in diagnosis. *J Acquir Immune Defic Syndr* 1991;4:250-3.
- Kovacs JA, Ng VL, Masur H, et al. Diagnosis of *Pneumocystis carinii* pneumonia: improved detection in sputum with use of monoclonal antibodies. *N Engl J Med* 1988;318:589-93.
- Ng VL, Yajko DM, McPhaul LW, et al. Evaluation of an indirect fluorescent-antibody stain for detection of *Pneumocystis carinii* in respiratory specimens. *J Clin Microbiol* 1990;28:975-9.
- Stratton N, Hryniewicki J, Aarnes SL, Tan G, de la Maza LM, Peterson EM. Comparison of monoclonal antibody and calcofluor white stains for the detection of *Pneumocystis carinii* from respiratory specimens. *J Clin Microbiol* 1991;29:645-7.
- Cregan P, Yamamoto A, Lum A, VanDerHeide T, MacDonald M, Pulliam L. Comparison of four methods for rapid detection of *Pneumocystis carinii* in respiratory specimens. *J Clin Microbiol* 1990;28:2432-6.
- Gill VJ, Evans G, Stock F, Parrillo JE, Masur H, Kovacs JA. Detection of *Pneumocystis carinii* by fluorescent-antibody stain using a combination of three monoclonal antibodies. *J Clin Microbiol* 1987;25:1837-40.
- Kovacs JA, Gill V, Swan JC, et al. Prospective evaluation of a monoclonal antibody in diagnosis of *Pneumocystis carinii* pneumonia. *Lancet* 1986;ii:1-3.
- Midgley J, Parsons P, Leigh TR, et al. Increased sensitivity of immunofluorescence for detection of *Pneumocystis carinii*. *Lancet* 1989;ii:1523.
- Birriel JA, Adams JA, Saldana MA, et al. Role of flexible bronchoscopy and bronchoalveolar lavage in the diagnosis of pediatric acquired immunodeficiency syndrome-related pulmonary disease. *Pediatrics* 1991;87:897-9.
- Rorat E, Garcia RL, Skolom J. Diagnosis of *Pneumocystis carinii* pneumonia by cytological examination of bronchial washings. *JAMA* 1985;254:1950-1.
- Pitchenik AE, Ganjei P, Torres A, Evans DA, Rubin E, Baier H. Sputum examination for the diagnosis of *Pneumocystis carinii* pneumonia in the acquired immunodeficiency syndrome. *Am Rev Respir Dis* 1986;133:226-9.
- Broadbent C, Dake MD, Stulberg MS, et al. Bronchoalveolar lavage and transbronchial biopsy for the diagnosis of pulmonary infections in the acquired immunodeficiency syndrome. *Ann Intern Med* 1985;102:747-52.
- Schumann GB, Swensen JJ. Comparison of Papanicolaou's stain with the Gomori methenamine silver (GMS) stain for the cytodagnosis of *Pneumocystis carinii* in bronchoalveolar lavage (BAL) fluid. *Am J Clin Pathol* 1991;95:583-6.
- Hopkin JM. *Pneumocystis carinii*. Oxford: Oxford University Press, 1991.
- Walzer PD, Perl DP, Krogstad DJ, Rawson PG, Schultz MG. *Pneumocystis carinii* pneumonia in the United States: epidemiologic, diagnostic and clinical features. *Ann Intern Med* 1974;80:83-93.
- Watts JC, Chandler FW. Evolving concepts of infection by *Pneumocystis carinii*. *Pathol Ann* 1991;26:93-138.
- Limper AH, Offord KP, Smith TF, Martin WJ. *Pneumocystis carinii* pneumonia: differences in lung parasite number and inflammation in patients with and without AIDS. *Am Rev Respir Dis* 1989;140:1204-9.
- Miller RF, Kocjan G, Buckland J, Holton J, Malin A, Semple SJG. Sputum induction for the diagnosis of pulmonary disease in HIV positive patients. *J Infect* 1991;23:5-15.
- Nelson M, Bower M, Smith D, Gazzard BG. Life-threatening complication of sputum induction. *Lancet* 1990;335:112-3.
- Hughes WT. *Pneumocystis carinii* pneumonia: new approaches to diagnosis, treatment and prevention. *Pediatr Infect Dis J* 1991;10:391-9.
- Miller RF, Millar AB, Semple SJG. Complications of fiberoptic bronchoscopy in HIV-1 antibody positive patients undergoing investigations for pulmonary disease. *Thorax* 1988;43:847.
- Griffiths MH, Kocjan G, Miller RF, Godfrey-Faussett P. Diagnosis of pulmonary disease in human immunodeficiency virus infection: role of transbronchial biopsy and bronchoalveolar lavage. *Thorax* 1989;44:554-8.
- Geelhoed GW. Open lung biopsy for the diagnosis of *Pneumocystis carinii* pneumonia. In: Robbins JB, De Vita VT, Dutz W eds. *Symposium on Pneumocystis carinii infection*. National Cancer Institute Monograph 43. Bethesda Maryland: DHEW, 1976:141-7.
- Craddock C, Pasvol G, Bull R, Protheroe A, Hopkin J. Cardiorespiratory arrest and autonomic neuropathy in AIDS. *Lancet* 1987;ii:16-8.
- Walzer PD. Diagnosis of *Pneumocystis carinii* pneumonia. *J Infect Dis* 1988;157:629-32.
- Baselski VS, Robison MK, Pifer LW, Woods DR. Rapid detection of *Pneumocystis carinii* in bronchoalveolar lavage samples by using cellufluor staining. *J Clin Microbiol* 1990;28:393-4.
- Walker J, Conner G, Ho J, Hunt C, Pickering L. Giemsa staining for cysts and trophozoites of *Pneumocystis carinii*. *J Clin Pathol* 1989;42:432-4.
- Linder J, Radio SJ. Immunohistochemistry of *Pneumocystis carinii*. *Semin Diagn Pathol* 1989;6:238-44.
- Naryshkin S, Daniels J, Freno E, Cunningham L. Cytology of treated and minimal *Pneumocystis carinii* pneumonia and a pitfall of the Grocott methenamine silver stain. *Diagn Cytopathol* 1991;7:41-7.

- 36 Lundgren B, Lipschik GY, Kovacs JA. Purification and characterization of a major human *Pneumocystis carinii* surface antigen. *J Clin Invest* 1991;87:163-70.
- 37 Peglow SL, Smulian AG, Linke MJ *et al*. Serologic responses to *Pneumocystis carinii* antigens in health and disease. *J Infect Dis* 1990;161:296-306.
- 38 Chatterton JMW, Joss AWL, Davidson MM, Ho-Yen DO. Why have *Pneumocystis carinii* trophozoites been ignored? *J Clin Pathol* 1990;43:265-8.
- 39 Tanabe K, Furuta T. Detection of circulating antigens of *Pneumocystis carinii* in human sera by a sandwich enzyme-immunoassay. *Zbl Bakt Hyg A* 1987;264:373-8.
- 40 O'Doherty MJ, Bateman NT. Prophylaxis and treatment of *Pneumocystis carinii* pneumonia. *Br J Hosp Med* 1991;45:277-83.
- 41 Phair J, Munoz A, Detels R, Kaslow R, Rinaldo C, Saah A. The risk of *Pneumocystis carinii* pneumonia among men infected with human immunodeficiency virus type 1. *N Engl J Med* 1990;322:161-5.
- 42 Graham NMH, Zeger SL, Park LP *et al*. Effect of zidovudine and *Pneumocystis carinii* pneumonia prophylaxis on progression of HIV-1 infection to AIDS. *Lancet* 1991;338:265-9.
- 43 Tanabe R, Fuchimoto M, Egawa K, Nakanura Y. Use of *Pneumocystis carinii* genomic DNA clones for DNA hybridization analysis of infected human lungs. *J Infect Dis* 1988;157:593-6.
- 44 Wakefield AE, Pixley FJ, Banerji S, *et al*. Detection of *Pneumocystis carinii* with DNA amplification. *Lancet* 1990;336:451-3.
- 45 Wakefield AE, Guiver L, Miller RF, Hopkin JM. DNA amplification on induced sputum samples for diagnosis of *Pneumocystis carinii* pneumonia. *Lancet* 1991;337:1378-9.